Review Article

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Inflammation & apoptosis in spinal cord injury

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Spinal cord injury (SCI) consists of a two-steps process involving a primary mechanical injury followed by an inflammatory process and apoptosis. Secondary insult is characterized by further destruction of neuronal and glial cells, and leads to expansion of the damage, so that the paralysis can extend to higher segments. With the identification of mechanisms that either promote or prevent neuronal inflammation and apoptosis come new approaches for preventing and treating neurodegenerative disorders. From a clinical perspective, this article discusses novel targets for the development of therapeutic agents that have the potential to protect the spinal cord from irreversible damage and promote functional recovery.

Key words Apoptosis - inflammation - mechanisms - spinal cord injury - therapy

Introduction

Spinal cord injury (SCI) leads to complex cellular and molecular interactions within the central nervous system in an attempt to repair the initial tissue damage[1]. The pathophysiology of SCI is characterized by the shearing of cell membranes and axons, disruption of the blood-spinal cord barrier, cell death, immune cell transmigration, and myelin degradation[2,3]. There are two mechanisms of damage to the spinal cord after injury: a primary mechanical injury and a secondary injury mediated by multiple injury processes including inflammation, free radical-induced cell death, and glutamate excitotoxicity[4]. The primary damage is locally restricted to the area of the vertebral fracture and is characterized by acute haemorrhage and ischaemia. Secondary insult within the first week after injury is characterized by further destruction of neuronal and glial cells, and leads to significant expansion of the damage, so that the paralysis can extend to higher segments. Deleterious factors, such as pro-inflammatory cytokines, proteases upregulated by immune cells and toxic metabolites, and neurotransmitters which released from lysed cells can induce further tissue damage.

While high dose methylprednisolone steroid therapy alone has not proved to be the solution to this difficult clinical problem, other strategies for modulating inflammation and changing the make up of inhibitory molecules in the extracellular matrix provided robust evidence that rehabilitation after SCI has the potential to significantly change the outcome for what was once thought to be permanent disability[5,6]. However, there has been no fully restorative therapy for SCI as yet and so prevention is the best medicine[7]. From a clinical perspective, this article provides processes of the secondary injury and the targets that have the potential

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to protect the spinal cord from irreversible damage and promote functional recovery.

Inflammation after SCI

Inflammatory responses are a major component of secondary injury and play a central role in regulating the pathogenesis of acute and chronic SCI, and seem to play a pivotal role in nerve injury and contribute to the control of the regenerative response[8]. Meanwhile, inflammatory responses may result in apoptosis of neurons and oligodendrocytes as well as in scar formation and finally in the reduction of neuronal function[9]. Therefore, it is believed that reducing inflammation could decrease secondary degeneration and the functional deficit after SCI.

Inflammatory reactions after SCI

After spinal cord trauma, ruptured blood vessels disturb the blood-brain barrier and the injury site is rapidly infiltrated by blood-borne neutrophils. This process may contribute to the secondary damage that follows the initial primary injury. At 30-45 min post-SCI, tumour necrosis factor (TNF)-positive cells could be seen over the injured spinal cord segment and from 3 to 24 h, TNF-α and interleukin-6 (IL-6) were strongly upregulated around the contused area[10,11]. The inflammatory cytokine mRNAs were shown to be induced as early as 15 min following contusion of rat spinal cord, with TNF-α increased first, followed by IL-6 mRNA[12,13]. TNF-α could potentiate glutamate-mediated neuronal cell death in the rat spinal cord[14,15], while TNF antagonist reduced the development of inflammation and tissue injury events associated with SCI[16,17]. Besides, IL-6 receptor monoclonal antibody treatment suppressed the astrocytic differentiation, decreased the number of inflammatory cells and the severity of connective tissue scar formation[18,19]. In studies, hyper-IL-6 infusion induced a six-fold increase in the number of neutrophils, a two-fold increase in the areas of spinal tissue occupied by macrophages and activated microglia and a four-fold decrease in axonal growth at the lesion site[20,21].

Increased production of cytokines of the IL-1 family, such as IL-1α, is well documented, providing clear evidence for a pivotal role of this cytokine in triggering SCI-induced inflammatory processes[22-24]. IL-1α and IL-18 are potent mediators of inflammation and initiate and/or amplify a wide variety of effects associated with innate immunity, host responses to tissue injury, and microbial invasion. Moreover, it has been speculated that the inflammasome is kept in an inactive state in normal tissues by binding to a putative caspase-1 inhibitor, but the nature of this inhibitor has not been described[25]. The study showed that a molecular platform (NALP1 inflammasome) consisting of NALP1, adipose-derived stem cell (ASC), caspase-1, and caspase-11 was present in neurons of the normal rat spinal cord and formed a protein assembly with the inhibitor of apoptosis family member, X-linked inhibitor of apoptosis protein (XIAP). And SCI induced rapid processing of IL-1α and IL-18, activation of caspase-1, cleavage of XIAP, and promoted assembly of the NALP1 inflammasome. Further, neutralization of ASC reduced caspase-1 activation and XIAP cleavage and decreased processing of IL-1α and IL-18, leading to improved histopathological and functional outcomes after SCI[26].

Central nervous system inflammatory responses that occur after SCI are initiated by peripherally derived immune cells, and activated glial cells that proliferate or migrate into the lesion site following injury[27]. T-cells are essential for activating macrophages and mounting a cellular or immune response. In rats, SCI activates myelin basic protein (MBP)-reactive T cells capable of causing neuron-inflammation and transient paralysis. In SCI, the frequency of MBP-reactive T cells increases, reaching levels that approximate those seen in multiple sclerosis (MS) patients. The pathogenic potential of SCI-activated B cells still remains to be directly tested, but early indications suggested that B cells also were pathological[16]. Data from other models also confirmed a direct link between primary CNS pathology and peripheral lymphocyte activation. Once lymphocytes gain access to the injury site, they persist indefinitely. Indeed, T and B cell numbers increase in the mouse SCI lesion through at least 9 wk post-injury[1]. Macrophages and neutrophils have also been proposed to participate in tissue destruction and enlargement of the lesion. Macrophages and microglia contribute to the secondary pathological and inflammatory response, in part through the release of cytokines, TNF, IL-1, IL-6, and IL-10[28], interferon, and activation of interleukin receptors (IL-4R and IL-2R). Cytokines facilitate CNS inflammatory responses by inducing expression of additional cytokines, chemokines, nitric oxide (NO), and reactive oxygen. Based on the presence and position of the first cysteine residues, the chemokines have been divided into four subgroups, *i.e.*, CC, XC, CX3C and CXC[29].

Molecules acting as anti-inflammatory agents

It has been indicated that in various types of injuries, some molecules act as anti-inflammatory agents and regulate invasive migration of immune cells to the site of damage. Many of anti-inflammatory agents could have potential also in the elimination of the secondary damage after SCI[6]. Based on the observation that the protective effects of gluococorticoids were independent on their receptors, a new group of steroid analogues, lazaroids, were developed. These analogues inhibit lipid peroxidation without glucocorticoid/mineralocorticoid activity and in such a way avoid the complications of steroid therapy. Accordingly, aminosteroid lazaroid U-74389G reduced the production of systemic and spinal IL-8 (neutrophile attractant and activator) as well as systemic interleukin-1 receptor antagonist (IL-1ra) after SCI induced by aortic cross-clamping. This report indicates that lazaroid may attenuate ischaemic endothelial cell injury or activation of leukocytes effect[30]. Moreover, neutrophil infiltration to the site of injured spinal cord could be eliminated by specific and potent neutrophil elastase inhibitor ONO-5046[31]. It was shown that spinal cord compression increased CINC-1 mRNA expression and protein synthesis. CINC-1 is a neutrophil chemoattractant and acute-phase protein induced by focal brain injury causing leukocyte mobilization and liver injury. This increase correlates with neurologic damage in injured rats. March *et al*[32] showed that ONO-5046 attenuated neurologic damage partly by blocking CINC-1 production. In addition, sphingosine-1-phosphate (Sph-1-P) could act as a specific and an effective motility regulator of human neutrophils. It was demonstrated that Sph-1-P inhibited trans-endothelial migration and invasiveness of neutrophils into human umbilical vein endothelial cells (HUVEC)-covered collagen layers, while no effect on their adhesion to HUVECs was observed. Although the mechanism of its action is not yet fully understood, this result indicates that Sph-1-P has the potential to be used as anti-inflammatory agent regulating invasive migration of neutrophils through endothelial layers at injured vascular site[22].

A novel phosphoprotein, proliferation related acidic leucine-rich protein (PAL31) has been identified in the nervous system, and its expression gradually declines with the developmental process and is rarely expressed in the adult nervous system, including the spinal cord. In addition to the function in proliferation, PAL31 could act as a caspase-3 inhibitor, which might negatively regulate the expression of macrophage chemoattractant protein 1 (MCP-1) and signal transducer and activator of transcription-1 (STAT-1) and rescue macrophages from apoptosis during an inflammatory response[33]. Besides, alleviation of this damage-induced signal in the repair-model SCI rat showed a good correlation with better recovery of damage spinal cords, and PAL31 might behave like an inflammatory modulator in response to the regeneration process in SCI rats[34]. Most interestingly, knockdown of PAL31 in macrophages triggered apoptosis in cells stimulated with interferon (IFN-γ) or lipopolysaccharide (LPS), which suggested that PAL31 might play an important role in maintaining the survival of macrophage in the presence of inflammatory stress[9].

Apoptosis after SCI

Apoptosis of targeted cells within a tissue is mediated by activation of cell signaling that results from either engagement of the apoptotic stimuli and cell surface death receptors or from direct disruption of the mitochondria and the subsequent activation of a proteolytic cascade involving executioner caspases[35-37]. In apoptosis, a biochemical cascade activates proteases that destroy molecules required for cell survival and others that mediate a programme of cell suicide. During the process, the cytoplasm condenses, mitochondria and ribosomes aggregate, the nucleus condenses, and chromatin aggregates[38]. Other features of apoptosis are reduction in the membrane potential of the mitochondria, intracellular acidification, generation of free radicals, and externalization of phosphatidylserine residues[39].

Apoptosis, as demonstrated by nuclear DNA fragmentation and caspase activation, was a prominent feature in the spinal cord post SCI. After SCI, some cells at the lesion site die by post-traumatic necrosis, whereas others die by apoptosis[40]. Apoptotic cell death was observed in both neurons and oligodendrocytes and was prominent in the white matter, in which wallerian degeneration was simultaneously observed. Thus, apoptosis of both neurons and oligodendrocytes may contribute greatly to the paralysis of patients with SCI[41,42].

Processes of apoptosis after SCI

A time course analysis in rats revealed that apoptosis occurred as early as 4 h post injury and could be seen in decreasing amounts as late as 3 wk after SCI[42]. After SCI, caspase activation occurs in neurons at the injury site within hours, and in oligodendrocytes adjacent to, and distant from, the

injury site over a period of days. The long-term neurological deficits after spinal cord trauma may be due in part to widespread apoptosis of neurons and oligodendroglia in regions distant from and relatively unaffected by the initial injury.

The major executioners in the apoptotic programme are proteases known as caspases[43]. The caspase family of cysteine proteases regulates the execution of the mammalian apoptotic cell death programme. Caspase-3 cleaves several essential downstream substrates involved in the expression of the apoptotic phenotype *in vitro*, including gelsolin, PAK2, fodrin, nuclear lamins and the inhibitory subunit of DNA fragmentation factor. Caspase-3 activation *in vitro* can be triggered by upstream events, leading to the release of cytochrome c from the mitochondria and the subsequent transactivation of procaspase-9 by Apaf-1. These upstream and downstream components of the caspase-3 apoptotic pathway are activated after traumatic spinal cord injury in rats, and occur early in neurons in the injury site and hours to days later in oligodendroglia adjacent to and distant from the injury site[44,45].

Caspase-8 and 9 are the initiator caspases in the death receptor and the mitochondrial dependent pathways, respectively, and their activation is a tightly regulated process[46]. Downstream effector caspases like caspase-3 are subsequently activated via proteolytic cleavage by these initiator caspases[47]. The inhibitor of caspase-activated deoxyribonuclease, the Bcl-2 family of proteins, cytoskeletal proteins like gelsolin, focal adhesion kinase and p21-activated kinase, and proteins involved in DNA repair, mRNA splicing and DNA replication[48,49] are some key proteins among the over forty target substrates for caspase-3 that have been identified to date.

Seminal studies have identified several genes that control cell death, in which four genes are required for the orderly execution of the developmental apoptotic programme, including ced-3 (caspases), ced-4 (Apaf-1), and egl-1 (BH3-only proteins)[50]. By contrast, ced-9 (Bcl-2) was indicated as an inhibitor of apoptosis[51].

Mediators of cellular apoptosis

SCI pathology results from complex interactions between different cell types and secreted molecules in a time-dependent manner. SCI leads to increased expression of death receptors and their ligands as well as activation of caspases and calpain.

Oxidants have, and continue to receive much attention as triggers of apoptosis. Studies have focused on the mechanisms by which H_2O_2 modulates the apoptotic pathway given the pivotal role that H_2O_2 plays in ischaemia/reperfusion injury to cerebral microvasculature and neuronal cells[52]. An integrated model of H_2O_2-mediated cellular apoptosis is unresolved although existing evidence implicates H_2O_2 in apoptosis initiation in both the mitochondrial and the death receptor signaling pathways. The more popular paradigm supports H_2O_2 as a mediator of mitochondrial membrane potential collapse that leads to the release of cytochrome c and the activation of caspase-9. Mitochondrial as well as extramitochondrial systems, such as cytoplasmic cytochrome P-450 and membrane bound NADPH oxidase are examples of physiologically relevant H_2O_2 sources[52].

The glutathione/glutathione disulphide (GSH/GSSG) redox system is a major contributor to the maintenance of the cellular thiol redox status. Evidence showed that decrease in cell GSH was associated with enhanced cellular apoptosis while increases in GSH were associated with expression of the anti-apoptotic protein, Bcl-2[53]. In more recent studies, they showed that it was the change in cellular GSH-to-GSSG ratio rather than changes in GSH *per se* that specifically mediated cell apoptosis and that this redox imbalance induced apoptosis was preceded by caspase-3 activation[54]. The two identified targets for redox control in apoptotic signaling are the mitochondrial permeability transition and caspases[35].

Current evidence shows TNFα, a proinflammatory cytokine which is best known for its role in immune and vascular responses, can induce apoptosis in non-immune tissues via the death domain of its cell surface receptor, TNF-R1. However, there are conflicting reports as to the role of cell death in SCI that probably reflect the known capacity of TNF to be both pro- and anti-apoptotic[54-56].

Fas-mediated neuronal and oligodendroglial apoptosis through the mitochondrial signaling pathway could be an important event that might ultimately contribute to demyelination, axonal degeneration and neurological dysfunction after SCI[57]. Preventing the activation of Fas-mediated cell death using neutralization of endogenous FasL is, therefore, a highly relevant neuroprotective approach, and warrants further investigation. Yu *et al*[58] showed that Fas-mediated apoptosis could be amplified by the intrinsic mitochondrial pathway after SCI.

Inhibitors of apoptosis

To control aberrant caspase activation, which can kill the cell, additional molecules inhibit caspase-mediated pathways. Among these are proteins known as inhibitors of apoptosis. These inhibitors interact directly with modulators of cell death. For example, the X-linked inhibitor of apoptosis and the neuronal inhibitor of apoptosis are proteins in neurons that directly inhibit caspase-3 activity and protect neurons from ischaemic injury[39,59].

The inhibitor of apoptosis protein (IAP) family of anti-apoptotic proteins, which are conserved across evolution with homologues found in vertebrate and invertebrate species, have a key function in the negative regulation of programmed cell death in a variety of organisms. Several mammalian homologues (XIAP, cIAP-1, cIAP-2, NAIP, Bruce, Survivin, and pIAP) have been identified, most of which have been demonstrated to inhibit cell death. Although the biochemical mechanism by which IAP-family proteins suppress apoptosis is controversial, at least some of the human IAPs (XIAP, cIAP-1, and cIAP-2) have been reported to directly bind and inhibit certain caspases, including caspases-3, -7 and -9. Thus, IAPs can inhibit caspases within both the death receptor and mitochondrial pathways. During apoptosis induced by the TNF family member Fas, XIAP is cleaved, separating the BIR1-2 domains from the BIR3-Ring domain. The BIR1-2 fragment is capable of inhibiting active caspases-3 and -7, but it is turned over rapidly in cells. Thus, cleavage of XIAP may be a mechanism for lowering the threshold of caspase activity necessary for inducing apoptosis[60].

Therapy

Since inflammation contributes to both constructive and neurodestructive processes, a more thorough understanding of the autoimmune events that occur following SCI may allow us to develop strategies that will harness the beneficial effects of inflammation and, hopefully, help to promote functional recovery. A number of experimental studies have been performed to establish a strategy for treatment of SCI, using surgical, pharmacological, and physiologic methods[61-64]. Although several chemical agents have been found to prevent neuronal tissue damage after SCI, a few can reduce the degree of neuronal damage or improve functional recovery after SCI. New methods of treatment of SCI that yield marked improvement of neurologic deficits without side effects are thus required[65,66].

Anti-inflammation strategies

Because multiple harmful substances are involved in the secondary SCI, it is unlikely that blocking one substance or mechanism would significantly prevent the course of secondary injury. Recently, several laboratories have shown remarkable protection and recovery of function in models of spinal cord injury using treatments that target components of the CNS inflammatory response.

A candidate molecule that could serve as a common or converging mediator for the secondary SCI is phospholipase A2 (PLA2)[67], which could be induced by multiple harmful substances including inflammatory cytokines, free radicals[68], and excitatory amino acids[69-71], and its metabolic products are involved in multiple injury processes. Meanwhile, increased levels of PLA2 and their metabolites may also induce inflammation, oxidation, and neuron toxicity[72], which could further exacerbate the injury.

Many of the pharmacologic approaches to SCI have been aimed at cell death caused by excitatory amino acids (EAAs). However, for a variety of possible reasons, clinical trials of EAA antagonists have not been efficacious[73]. The recently reported studies illustrate new therapeutic approaches that might be effective, at least in part, by interfering with the acute CNS inflammatory cascade. It was shown that injury induced the immune receptor CD95 and that blockade of this receptor produced a better recovery after experimental SCI in mice[74]. Meanwhile, an antibiotic tetracycline derivative, minocyline, that has anti-inflammatory and anti-apoptotic actions provided substantial sparing of both neurons and glial cells and also resulted in better neurological outcomes in two different SCI models in rats[75-77]. Another recent paper reports a novel effective treatment that reduces the NO release that is associated with acute inflammation after SCI[78]. The effects on apoptosis were manifest hours to days later, but there were also effects on lesion size, which were perhaps due to reductions in acute necrotic cell death[79]. The results of each study indicated a reduction of axonal 'dieback' and hinted at enhanced regeneration.

Lipid peroxidation (LP) is one of the most harmful mechanisms developed after SCI. Several strategies have been explored in order to control this phenomenon. Protective autoimmunity (PA) is in fact a new concept that refers to an innovative approach where autoreactive mechanisms are modulated in order to promote neuroprotection. In light of this concept,

immunization with neural-derived antigens has shown to modulate this autoreactive response and to render protective and restorative effects after SC injury[80-82]. For instance, a study demonstrated that immunization with neural-derived peptides induces a better motor recovery compared to control animals[83]. The same study showed that this strategy improves neuronal survival and myelin preservation in SC-injured rats[84,85].

The use of minocycline, an antibiotic that reduces microglial activation, antibody blockade of the CD95 (FAS) ligand and the blockade of glycosphingolipid-induced iNOS (inducible nitric oxide synthase) has recently been shown to reduce neuronal and glial apoptosis with concomitant improvement in neurological function, and to enhance the efficacy of cell transplantation strategies[79]. Dexmedetomidine is a highly selective and potent adrenergic agonist that is increasingly being used as an adjunct for general anaesthesia[86,87]. Use of dexmedetomidine as an anaesthetic adjunct does not change somatosensory or motor-evoked potential responses during complex spine surgery by any clinically significant amount[88]. Dexmedetomidine is found to be safe and effective in various neuraxial and regional anaesthetics in humans[89,90]. Methylprednisolone is often used in the setting of acute SCI with anti-inflammatory properties that were thought to reduce spinal cord oedema[91].

Anti-apoptotic strategies

Better understanding of the molecular and cellular mechanisms of neuronal apoptosis has led to the identification of specific drug targets. The short-term necrotic damage seems to set up the conditions for longer-term apoptosis in a way that reflects the pattern of axonal loss[92,93].

One approach is to block the apoptotic trigger, and other approaches target early premitochondrial alterations, such as drugs that scavenge free radicals, block calcium influx into neurons or inhibit the activity of Par-4[94]. Activation of anti-apoptotic pathways by treatment with neurotrophic factors is another approach. Moreover, within the nervous system, IAPs have been shown to protect some types of neurons from insults often associated with ischaemia. Virus-mediated overexpression of NAIPor XIAP can prevent ischaemic neuronal loss in the hippocampus. Conversely, in severe spinal muscular atrophy the neuron-specific inhibitor of apoptosis, NAIP, is often dysfunctional due to missense and truncation mutations, suggesting that NAIP mutations may alter development of sensory and motor systems resulting in lethal muscular atrophy[95]. Excitatory amino acids appear to act on surviving neurons and oligodendrocytes and to promote autodestructive changes after SCI. The findings indicated that the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 could reduce apoptosis and reverse motor impairment following SCI[96].

Therapy of stem cells and genes

The functions of stem cell factor in the nervous system have not yet been fully elucidated, while the patterns of expression of both stem cell factor and c-kit have been well studied. It has recently been reported that stem cell factor functions as a survival factor for neural stem cells *in vitro*[97]. The study found that upregulation of stem cell factor and c-kit expression occurred after SCI, and that stem cell factor administration prevented neuronal cell apoptosis after SCI. Meanwhile, cytokines could play an important role in the signal network of an inflammatory response in tissue scar formation following SCI[98]. Pearse *et al*[92] found that increases in cAMP enhanced the efficacy of Schwann cell transplants on recovery, but only if the cAMP levels were increased acutely after injury. And cAMP given acutely dramatically reduced the production of the inflammatory cytokine TNF-α.

Further, micro RNA (miRNA) and small interfering RNA (siRNA) mediated RNA interference (RNAi) is considered to be a valuable tool for silencing of each gene in eukaryotes in post-transcriptional manner. Wu *et al*[99] successfully used plasmids containing pre-miRNA sequences to knock-down the CCR1 gene expression in MCCLM3 cells resulting in inhibition of cell invasion. Similarly Miyazaki *et al*[100] showed that downregulated CXCL5 expression using RNAi decreases proliferation and invasion ability of squamous cell carcinoma. In spite of the fact that RNAi was already used to reduce the expression of chemokine genes as well as genes of chemokine receptors, this approach was not yet used in the field of SCI research[101,102].

Conclusion

This review has discussed the major issues associated with inflammatory process and apoptosis in spinal cord injury. SCI is a devastating condition for which there is as yet no cure. With the identification of mechanisms that either promote or prevent spinal cord inflammation and apoptosis come new approaches for preventing and treating spinal cord injury. And an understanding of the basic secondary pathophysiologic processes outlined above provides the basis for current

therapy, and in addition, provides a framework for the development of new treatment strategies. Meanwhile, cellular, molecular and rehabilitative training therapies are being developed and some are now in, or moving towards, clinical trials. Nevertheless, work remains to be done to ascertain whether any of these therapies can safely improve outcome after human SCI.

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